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Evolutionary Implications of FGF and Distal-Less Expressions During Proximal-Distal Axis Formation in the Ampulla of a Direct-Developing Ascidian, *Molgula pacifica*

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The present results provide the first evidence of a fibroblast growth factor (FGF) family protein in a urochordate. Anti-FGF2 immunoreactive hemoblast cells were detected at day 3 of juvenile development in a direct-developing urochordate ascidian, Molgula pacifica. *The detection of FGF in hemoblast cells coincided with the appearance of distal-less protein along the proximal-distal axis of growing ampullae. Ampullae are limb-like, fluid-filled ectodermal appendages that contain hemoblast cells and have holdfast, respiratory, and immunological functions (1). Given the evolutionary conservation of the genes encoding FGF (2), their receptors (2), and distal-less (3), the present results suggest that the formation of non-homologous ascidian appendages shares genetic elements in common with proximal-distal axis formation in arthropod limbs and vertebrate limbs (4,5). The possible evolutionary implications of these findings are discussed.*

Members of the fibroblast growth factor (FGF) family of proteins have multiple functions in organizing the patterns of diverse kinds of tissues.[2] One member of this family, FGF2, has been implicated in mesoderm induction in amphibians (6) and in the initiation of limb development in chick embryos (7) and in mouse embryos (8,9). FGF2 does not function alone, but acts with other regulatory molecules including Wg/Wnt (7). In *Drosphilia,* it was recently reported that Wg can induce distal-less, a highly conserved homeoprotein, during the proximal-distal outgrowth of a fly's leg (4). Distal-less is a highly conserved gene that functions in the development of diverse kinds of appendages from onychophoran lobopods to mammalian limbs, and this gene is thought to have evolved in the pre-Cambrian ancestor of protostomes and deuterostomes (3).

In the present study, juveniles of the invertebrate ascidian chordate *Molgula pacifica* (belonging to the subphylum Urochordata), in which five ectodermal ampullar appendages grow out from the body wall of an early juvenile, were examined for two proteins that are known to be involved in the outgrowth of arthropod and vertebrate limbs. Histological sections of *M. pacifica* juveniles were stained with FGF2 antibody at days 2, 3, 4, and 5 of development. Anti-FGF2 staining was first observed at day 3 of development, exclusively in newly formed hemoblast cells, and the detection of these cells coincided with the appearance of distal-less proteins expressed along the proximal-distal axis of growing ampullae (Fig. 1A, B; Table I).

Prior to hemoblast cell formation, anti-FGF2 stained cells were not detected (50 serially sectioned juveniles were examined), and anti-EGF immunoreactive cells were not observed at day 2 or day 5 of development (30 serially sectioned juveniles were examined). Anti-FGF2 staining intensified in a subpopulation of hemoblast cells at days 4 and 5 of development (Fig. 1C). At day 5, the average number of hemoblast cells stained with FGF2 antibody was three to four cells per juvenile (a total of 132 juveniles were serially sectioned and stained with FGF2 antibody).

This is the first report in which an endogenous growth factor belonging to the FGF superfamily has been detected in a urochordate. Furthermore, the present results demonstrate that anti-FGF2 staining is restricted to hemoblast cells. In an indirect-developing ascidian, hemoblast

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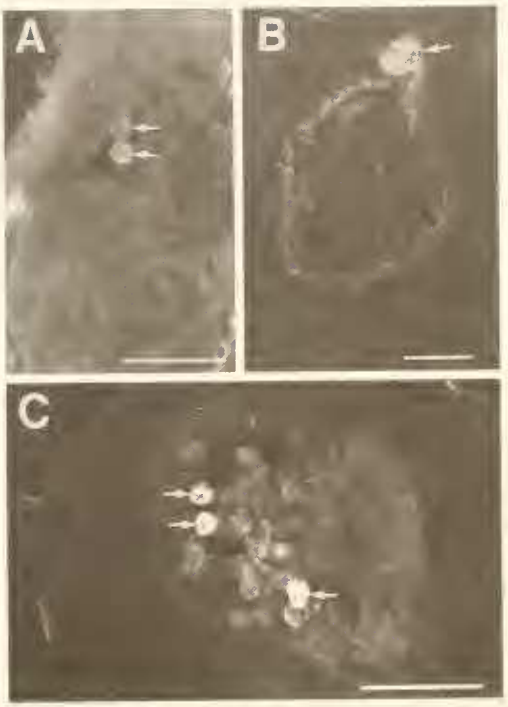

Figure 1. Immunocytochemical detection of FGF and distal-less proteins during the proximal-distal outgrowth of *Molgula pacifica* ampullae. *M. pacifica* adults were collected by scuba in the Barkley Sound region located along the west coast of Vancouver Island, British Columbia, Canada, near Bamfield Marine Station, Bamfield, British Columbia. Sperm and oocytes were obtained by the dissection of gonads. Oocytes were washed with large volumes of seawater and allowed to undergo germinal vesicle breakdown in seawater prior to their insemination with non-self sperm. Fertilized eggs were washed with large volumes of seawater and cultured at 11°C in glass Syracuse dishes on sea tables containing fresh, flowing seawater. *M. pacifica* juveniles at days 2, 3, 4, and 5 of development were fixed for 20 minutes in absolute methanol at −20°C followed by a 20-minute immersion in absolute ethanol at −20°C and then embedded in polyester wax for sectioning. Embedded specimens were cut into 8-μm-thick sections and floated on distilled water droplets placed on gelatin-coated shards of glass. After the sections dried overnight at room temperature, they were de-waxed and equilibrated with phosphate buffered saline (PSB) by immersing the shards with adhered sections through a graded series of ethanol followed by three washes with PBS. Prior to immersion into antibody solutions, sections were immersed in a blocking solution (1% BSA (Sigma Chemical Company) in PBS) for 30 min at room temperature. Shards with adhered sections were transferred to a moist chamber for antibody incubations. Anti-bovine FGF2 antibody was purchased from Sigma Chemical Company and diluted 1:60 with PBS containing 0.5% BSA. Sections stained with primary antibody for 1 h at room temperature in a moist chamber were immersed in FITC-labeled anti-rabbit secondary antibody diluted 1:60 with PBS. Bovine FGF2 was purchased from Sigma Chemical Company to preabsorb anti-FGF2 prior to immunochemistry for a control experiment. Sections probed with anti-FGF2 that was preabsorbed with FGF2 showed no fluorescence. Epidermal growth factor antibody was purchased from Sigma Chemical Company and diluted 1:60 with PBS containing 0.5% BSA. EGF incubation times and detection methods were the same as those used for the detection of FGF2. The distal-less antibody, kindly provided by Dr. Grace Panganiban, was used as previously described by Panganiban *et al.* (3), except that the specimens were fixed in methanol and ethanol, sectioned, and the primary antibody was detected using FITC-secondary antibody. Fluorescence was not detected when sections were incubated in secondary antibody without prior treatment with the primary antibody.

(A) Sections through a day 3 juvenile showing anti-FGF2 immunoreactive hemoblast cells (see arrows) situated within the hemocoel and (B) a day 3 ampulla (see arrow) stained with distal-less antibody. (C) Section through a day 5 juvenile showing intense anti-FGF2 stained hemoblast cells (see arrows). Scale bars in each frame equal 50 μm.

Table I

Hemoblast cell-specific immunoreactivity using anti-FGF2 in Molgula pacifica *juveniles*

Antibody	Stage (Day)	Cell type* and staining†				
		me	tu	ae	hb	ccm
FGF2	2	−	−	−	−	−
	3	−	−	−	+	−
	4	−	−	−	++	−
	5	−	−	−	+++	−
EGF	2	−	−	−	−	−
	5	−	−	−	−	−

* Me, mantle epidermis; tu, tunic; ae, ampullar epidermis; hb, hemoblast cells; ccm, central cell mass.

† −, unstained; +, weak staining; ++, intermediate staining; +++, intense staining.

cells were shown to be derived from larval trunk lateral cells (TLCs), decendents of the A7.6 lineage of a 110-celled embryo (10). Cell lineage studies, now in progress, will examine whether the anti-FGF2 immunoreactive hemoblast cells are derived from the A7.6 lineage or from another cell lineage of *M. pacifica* embryos.

The results of a previous experiment suggest that FGF may play a role earlier in ascidian development (11). When notochord progenitor cells were isolated from embryos and then immersed in FGF2, some of the descendants of these cells expressed a notochord-specific protein, termed "Not-1." Although these results suggest that FGF2 receptors may be present in notochord cells of indirect-developing embryos, it was not reported whether endogenous FGF2 actually exists in the embryo at the time of notochord induction. In *M. pacifica*, detailed light and electron microscopy studies failed to detect the presence of a notochord placode (12,13). The absence of this structure suggests that notochord cell fates were eliminated in this species, an event that may have been caused by a modification of an FGF-mediated inductive interaction, if it is assumed that the ancestor of *M. pacifica* was an indirect-developer.

Several intriguing evolutionary questions are raised by the present results. What are these conserved regulatory proteins for limb development doing in presumably non-homologous, limb-like ampullae? One possibility is that urochordate FGF and distal-less proteins, along with other limb-patterning regulatory proteins, repre-

sent some of the ancestral vertebrate genetic elements that are utilized in a modified manner during the development of a complex vertebrate limb. As previously described in detail (1), hemoblast cells are pumped into the lumen of growing ampullae by rhythmic contraction waves generated by the ampullar walls, and hemoblast cells are often observed in the tip region of ampullae. These contractions may bring FGF into close proximity with target FGF receptors that may be present on ampulla ectoderm cells. FGF may function as a mitogen along with distal-less and other regulatory proteins to promote ampullar outgrowth and patterning. A direct comparison can be made with limb development in salamanders. Mullen *et al.* (14) showed that the apical ectodermal cap cells of a salamander limb transcribe large amounts of Dlx3, a homolog of distal-less, and that Dlx3 expression is regulated by FGF2.

A second possibility is that evolutionary co-option for ampulla expression may have occurred in that FGF and distal-less proteins were appropriated for functions other than limb formation during their evolution. The resolution of these questions may be possible when urochordate FGF and distal-less genes are characterized and their signaling pathways, which likely involve other limb-patterning genes, are better understood. In conclusion, the expression of two conserved limb-patterning proteins, FGF and distal-less, during the outgrowth of ascidian ampullae represents an exciting experimental system for investigating the evolutionary development of animal appendages.

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